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TITLE: Molecular Differentiation of Risk for Disease Progression: Delineating Stage-Specific Therapeutic Targets for Disease Management in Breast Cancer

PRINCIPAL INVESTIGATOR: Maria J. Worsham, Ph.D.
Usha Raju, M.D.
Gary Chase, Ph.D.

CONTRACTING ORGANIZATION: Henry Ford Health System
Detroit, Michigan 48202

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6. AUTHOR(S)
Maria J. Worsham, Ph.D.
Usha Raju, M.D.
Gary Chase, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry Ford Health System
Detroit, Michigan 48202

E-Mail: mworsha1@hfhs.org

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Cancer is a highly heterogeneous disease, both morphologically and genetically. A current shortcoming in cancer prognosis and treatment is a lack of methods that adequately address the complexity and diversity of the disease. Genome wide studies can provide molecular characterization or fingerprints of cancer phenotypes linked to clinical information. The aim of this research is to **1a:** identify an informative set of specific genetic alterations that underlie the pathogenesis of disease progression to serve as targets for management of disease at the earliest stages and **1b**: refine stage-specific disease phenotypes by integration of molecular profiles with known risk factors of breast cancer such as reproductive characteristics, medical history, and histologic parameters of breast carcinomas. We will examine 100 cases in each disease stage category of 0, 1, 2, 3, 4 and unknown to evaluate 120 breast cancer associated gene markers distributed throughout the human genome. Molecular fingerprints identified from genome wide studies should delineate patterns of genomic imbalances at the level of stage -specific gene loci, providing a novel index to estimate the extent of genomic abnormality with disease progression. This knowledge should allow the integration of stage-specific therapeutic targets as treatment intervention strategies in the management of breast cancer.

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disease progression, genome wide, gene loci alterations, stage-specific markers, breast cancer

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INTRODUCTION

Cancer is a highly heterogeneous disease, both morphologically and genetically (1). A current shortcoming in cancer prognostication and treatment is a lack of methods that adequately address the complexity and diversity of the disease. A detailed molecular characterization or fingerprint of cancer is an objective recently made possible by the development of several new high throughput analytical methods. These include techniques for the analysis of DNA, mRNA, and proteins within a cell (2-4). Building databases of detailed molecular information and linking them to clinical information are very attainable goals (5). This approach has the potential to help patients by improving grouping of tumor subtypes, which may enable clinicians to more accurately distinguish prognostic groups, and predict the most effective therapies. Prognostic marker systems based on single parameters have generally proven inadequate. Thus, multiparametric methods, which rely on many pieces of information, are ideally suited to the grouping of tumor subtypes and the identification of specific patterns of disease progression.

A major objective of current cancer research is to develop a detailed molecular fingerprint of tumor cells and tissues that is linked to clinical information. Toward this end, using the Multiplex Ligatable Probe Amplification technique (MLPA, 6), a novel assay recently developed at MRC Holland (Amsterdam) we will interrogate 120 gene loci (Table 1, Study Instruments) altered in breast cancer using a nested case cohort of 600 stage-specific breast cancers drawn from a retrospective cohort of 6000 primary breast cancers.

REFERENCES

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2. Pollack J. R., Perou C. M., Alizadeh A. A., Eisen M. B., Pergamenschikov A., Williams C. F., Jeffrey S. S., Botstein D., Brown P. O. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat. Genet., 23: 41-46, 1999

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4. Oh J. M., Hanash S. M., Teichroew D. Mining protein data from two-dimensional gels: tools for systematic post-planned analyses. Electrophoresis, 20: 766-774, 1999

5. Golub T. R., Slonim D. K., Tamayo P., Huard C., Gaasenbeek M., Mesirov J. P., Coller H., Loh M. L., Downing J. R., Caligiuri M. A., Bloomfield C. D., Lander E. S. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science (Washington DC), *286:* 531-537, 1999

6. MRC Holland: Dr. J. P. Schouten, mrch@bio.vu.nl. Website: www.MRC-Holland.com

BODY: Statement of Work

Task 1. Cohort construction, Months 1-24

a: Begin construction of the breast cancer study cohort. We have identified 6000 breast cancer cases in the HFHS system from 1981 through 2000. Drs. Worsham and Chase will select 100 stage-specific breast cancers corresponding to stage 0 (in situ), stage 1, 2, 3, 4 and unknown stage

b: Set up database of study cohort

c: Retrieval of H & E slides for cancer cohort

d: The Pathologist Dr.Raju and the P.I will begin pathology review of the cancer cohort recording histopathological characteristics on the Pathology Cancer Review Form (see study instruments).

e: Data entry of histopathology indicators

f: selection of tumor blocks and sectioning of tissue for microdissection and DNA extraction

Task 2. Molecular Assays Months 3-34

a: Begin the novel Multiplex Ligatable Probe Amplification(MLPA) assays
b: As DNA becomes available set up molecular worksheets and forms for electronic data entry of molecular data (Teleform)

Task 3. Medical chart abstraction Months 3-32
a: Begin medical chart abstraction using the Medical record Abstraction Form
b: Data entry of forms into the study database

Task 4. Interim Analyses, Months 18-24

a: Interim statistical analysis of data obtained from molecular, pathology, and medical record abstractions will be performed periodically
b: Annual reports will be written

Task 5. Final Analyses and Report Writing, Months 32-36
a: Final analyses of data form molecular, pathology and medical abstractions will be performed
b: A final report and initial manuscripts will be prepared

PROGRESS (June 1, 2002- June 30, 2003:

KEY RESEARCH ACCOMPLISHMENTS

Task 1 accomplishments:

- We have so far acquired a total breast cancer patient database of 5008 validated and verified breast cancer cases. The study cohort of 600 stage-specific breast cancer subjects was derived from this comprehensive patient database. Selection of breast cancer subjects in each of the 6 stages, stage 0, stage 1, stage 2, stage 3, stage 4, and stage unknown was performed by the biostatistician Dr. Gary Chase. Criteria for selection were as follows: 1) age <50 years, Caucasian Americas (CA); age <50 years, African American (AA); 2) age >50 years, CA; age >50 years, AA. A total of 1,244 subjects were obtained as a result of this selection; stage 0=215; stage 1= 225; stage 2= 228; stage 3= 188; stage 4= 179; stage unknown= 209. Further selection of 100 stage-specific cases for equal representation of CA and AA in each of the two age categories, <50 years and > 50 years was achieved in a random fashion by Dr. Gary Chase. Thus, the study cohort of 600 breast cancer subjects, 100 in each of the 6 stages has been completed.
- Data bases of the study cohort have been completed and linked with the Henry Ford Health System Tumor Registry for demographics, histopathology, and clinical information. The latter has been obtained for the entire cohort of 1,244 subjects.
- The study Pathologist Dr. Raju has completed review of 210 breast cancer subjects
- Pathology Review Form data via electronic Teleform data entry has been entered for 210 study subjects
- Tissue block retrieval, sectioning, H & E staining, microdissection, and DNA extraction has been accomplished for 160 subjects

Task 2

- Multiplex Ligatable Probe Amplification (MLPA) assays have been performed for 120 subjects for a total of 309 lesions.

Task 3

- Medical record abstraction has been completed for 120 subjects and entered into the database

Progress: PENDING

Statistical analysis of the MLPA data for the 120 subjects with MLPA is awaiting completion.

REPORTABLE OUTCOMES

Abstract presented/Manuscripts

Preface:

Validation of the MLPA assay methodology, interpretation and data analysis was conducted in two separate cohorts:

1: MCF-10A model of breast cancer progression

2: Squamous head and neck cancer cohort

1A: Worsham MJ, Pals, F, Schouten JP, Miller F, von Spaendonk R, Wolman SR. High-resolution mapping of molecular events associated with immortalization and progression of breast cancer in the MCF10A model. *American Association of Investigative Pathology, San Diego, April 11-16, 2003.* The manuscript (in preparation) will be submitted to Cancer Research

ABSTRACT

A comprehensive and consistent picture of the genetic changes that underlie breast cancer initiation, development, and progression remains unresolved. The MCF10 series of cell lines represent many steps in that progression. We evaluated MCF10 cells in a series that included initial untransformed outgrowths (MCF-10MS and MCF10A) and 6 transformed cell lines recapitulating various stages of benign proliferation (MCF10-AT1, MCF10AT1kcl2), carcinoma in situ (MCF10CA1h cl13), and invasive carcinoma (MCF10CA1h cl2, MCF10CA1d cl1, MCF10CA1a cl1). Losses and gains of loci at 112 human genome sites using a novel assay called the multiplex ligation-dependent probe amplification assay (MLPA). Cytogenetic alterations in the four benign progenitors that persisted in the CIS and invasive cell lines, were validated by corresponding gains and losses of gene loci by MLPA. MCF-10MS had only normal gene copies. The untransformed MCF10A, resulting from spontaneous immortalization of MCF-10MS and the parent of subsequent transformed progeny had cytogenetic gain of 5q13-qter with corresponding gains of IL3, IL4 and IL12B gene loci at 5q31-q33; gain of distal 19q12-qter was validated by gains in KLK3 and BAX gene loci at 19q13-q13.4. Genic gain of cMYC at 8q24.12 was not indicated by cytogenetics. The apparently balanced t(3;9) component of the t(3;9)(p13;p22)t(3;5)(p26;q31) resulted in complete loss of the CDKN2A and CDKN2B loci. Additional clonal cytogenetic changes in the DCIS cell line (MCF10A1h cl13) involving chromosomes 1, 3 and 10 persisted in the invasive progeny, with gain of corresponding gene loci at 1p13 (BCAR2, BCAR3, NRAS, TGFB2), at 3p12-13 (IL12A), and 3q21-q27 (MME, PIK3CA, BCL6).

SUMMARY OF RESULTS

- Cytogenetic alterations in the four benign progenitors that persisted in the CIS and invasive cell lines were validated by corresponding gains and losses of gene loci by MLPA.
- The apparently balanced t(3;9) component of the t(3;9)(p13;p22)t(3;5)(p26;q31) resulted in complete loss of the *CDKN2A* and *CDKN2B* loci.
- Genic gain of *cMYC* at 8q24.12 was not indicated by cytogenetics.
- Immortalization is marked by complete loss of *CDKN2A/2B*, gain of c*MYC*, *IL3*, *IL4*, *IL12B*, *KLK3* and *BAX* gene loci.
- Events of progression involve further gain of *BCAR2*, *BCAR3*, *NRAS*, T*GFB2* (1p13), *IL12A* (3p12-13), *MME*, *PIK3CA*, *BCL6* (3q21-q27), and *NCO3*.

1B: Worsham MJ, Pals, F, Schouten JP, Miller F, von Spaendonk R, Wolman SR. Genetic targets of immortalization in the MCF-10A model: High resolution mapping of loss of gene architecture at the *MTAP*, *CDKN2A*, and *CDKN2B* genetic loci. *Late-breaking Abstract, invited, American Association for Cancer Research, July 11-15, 2003, Washington D.C.* The manuscript will be submitted to Clinical Cancer Research

ABSTRACT

The MCF10 series of cell lines represents many steps in breast cancer initiation, development, and progression. MCF10A cells are spontaneously immortalized human breast epithelial cells derived from a sample of fibrocystic disease. We evaluated MCF10A cells in a series that included initial untransformed outgrowths (MCF-10MS and MCF10A) and 6 transformed cell lines recapitulating various stages of benign proliferation (MCF10-AT1, MCF10AT1kcl2), carcinoma in situ (MCF10CA1h cl13), and invasive carcinoma (MCF10CA1h cl2, MCF10CA1d cl1, MCF10CA1a cl1). All were characterized cytogenetically. The untransformed MCF10A, resulting from spontaneous immortalization of MCF-10MS (46,XX) has a karyotype of 46,XX,t(3;9)(p13;p22)t(3;5)(p26;q31),t(3;17)(p13;p12),der(6)t(6;19)(p25;q12). Subsequent transformed progeny are all karyotypically related.

DNA from MCF10-MS and MCF10A and the 6 transformed cell lines were interrogated for losses and gains of loci at 112 human genome sites using a novel assay called the multiplex ligation-dependent probe amplification assay (MLPA). This comprehensive genome-wide approach to localize and identify key genetic loci in the immortalization and evolution of breast cancer indicated normal copy number for all gene loci tested by the MLPA assay for MCF-10MS concordant with a normal 46, XX karyotype. Homozygous loss of the *CDKN2A* and *CDKN2B* genes at 9p21in immortalized MCF10A modified interpretation of the apparently balanced t (3;9)(p13;p22) translocation, altering the translocation breakpoint to 9p21 instead of 9p22.

A targeted MLPA probe set was designed to further refine the precise region of loss at the 9p21 locus. The high-resolution 9p probe set comprised ten probes for *CDKN2B*^{INK4b} (three p15 ^{INK4b} probes: start of exon 1, end of exon 1, end of exon 2) and *CDKN2A* $^{ARF, INK4a}$ genes (7 probes: start of exon 1□ [p14 ^{ARF}], exon 1□ [p14^{ARF}], 2 intron probes between p14^{ARF} and p16 ^{INK4a}, exon 1□ [p16 ^{INK4a}], exon 2 [p16^{INK4a}], exon 3 [p16 ^{INK4a}]), in addition to 3 probes for *MTAP*, two probes telomeric to 9p, and 15 other autosomal probes. All ten *CDKN2B* and *CDKN2A* probes validated homozygous loss of *CDKN2B* ^{INK4b} and *CDKN2A* $^{ARF, INK4a}$. Regional loss at 9p21 extended to include the distal end of the *MTAP* gene located proximal to the *CDKN2A* ^{INK4a} locus. Probes distal to *CDKN2B* in the subtelomeric region of 9p were retained. Other autosomal probes showed normal copy numbers or copy number changes consistent with cell line karyotypes. Identical loss of gene architecture along *CDKN2B*, *CDKN2A* and *MTAP* gene loci was confirmed for all subsequent transformed cell lines. In summary, loss of *CDKN2A* ^{ARF,INK4a}, *CDKN2B*, ^{INK4b} and *MTAP* genes are very early targets of immortalization in the MCF-10A model, defining a primary repertoire of genetic alterations along a molecular continuum in the progression of breast cancer.

RESULTS AND CONCLUSION

All ten *CDKN2B* and *CDKN2A* probes validated homozygous loss of *CDKN2B* ^{INK4b} and *CDKN2A* $^{ARF, INK4a}$. Regional loss at 9p21 extended to include the distal end of the *MTAP* gene located proximal to the *CDKN2A* ^{INK4a} locus. Probes distal to *CDKN2B* in the subtelomeric region of 9p were retained. Other autosomal probes showed normal copy numbers or copy number changes consistent with cell line karyotypes. Identical loss of gene architecture along *CDKN2B*, *CDKN2A* and *MTAP* gene loci was confirmed for all subsequent transformed cell lines. Identical loss of gene architecture along *CDKN2B*, *CDKN2A* and *MTAP* gene loci observed in untransformed MCF10A was confirmed in all subsequent transformed cell lines. In summary, loss of *CDKN2A* ^{ARF,INK4a}, *CDKN2B*, ^{INK4b} and *MTAP* genes are very early targets of immortalization in the MCF-10A model, defining a primary repertoire of genetic alterations along a molecular continuum in the progression of breast cancer.

2: Worsham MJ, Pals G, Shouten J, von Spaendonk R, Concus AP, Carey TE, Benninger MS. Delineating Genetic pathways of disease Progression in HNSCC. Arch of Otolaryngol Head and Neck Surg, 129: 699-701, July, 2003 (See appendix)

Manuscripts ready for submission (validation of novel MLPA technology):

a: Worsham MJ, Pals, F, Schouten JP, Miller F, von Spaendonk R, Wolman SR. High-resolution mapping of molecular events associated with immortalization and progression of breast cancer in the MCF10A model.

b: Worsham MJ, Pals, F, Schouten JP, Miller F, von Spaendonk R, Wolman SR. Genetic targets of immortalization in the MCF-10A model: High resolution mapping of loss of gene architecture at the *MTAP*, *CDKN2A*, and *CDKN2B* genetic loci.

APPENDICES

A: Study Instruments

a: Pathology Review Form: Teleform Version for Data Entry

b: MLPA Access Data Report Form

B: Publications (validation of novel MLPA technology):

Worsham MJ, Pals G, Shouten J, von Spaendonk R, Concus AP, Carey TE, Benninger MS.Delineating Genetic pathways of disease Progression in HNSCC. Arch of Otolaryngol Head and Neck Surg, 129: 699-701, July, 2003

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CANCER PATHOLOGY REVIEW FORM (page1)

MRN ☐☐☐☐☐☐☐

Pathology_No ☐☐☐☐☐☐☐☐

Path Report Date ☐☐/☐☐/☐☐☐☐

Type of tissue sample

- ○ Needle Biopsy
- ○ Excision Biopsy
- ○ Simple Mastectomy
- ○ Modified Radical Mastectomy
- ○ Other
- ○ Unknown

Stage

- ○ 0
- ○ 1 ○ 1A ○ 1B ○ 1C
- ○ 2 ○ 2A ○ 2B
- ○ 3 ○ 3A ○ 3B
- ○ 4
- ○ Unknown

Source of Breast Tissue

- ○ Left
- ○ Right
- ○ Unknown

Size of Largest Mass (cm) ☐☐.☐☐

MICROSCOPIC FINDINGS

MALIGNANCY **Present** ○ No ○ Yes

Foci ○ 1 ○ >1 ○ >multifocal

Block1 ☐☐☐ **Block2** ☐☐☐

Block3 ☐☐☐ **Block4** ☐☐☐

Block5 ☐☐☐ **Block6** ☐☐☐

Type ○ Invasive ductal ○ Invasive lobular ○ Tubular ○ Mucinous ○ Medullary ○ Inflammatory ○ Other

Carcinoma In Situ

○ No ○ Lobular ○ Ductal ○ Both

Block1 ☐☐☐ **Block2** ☐☐☐ **Block2** ☐☐☐

Grade ○ 1 ○ 2 ○ 3 **Nuclear** ○ 1 ○ 2 ○ 3

Angiolymphatic Invasion ○ No ○ Yes

Necrosis ○ No ○ Yes

Grade

Nuclear ○ 1 ○ 2 ○ 3 ○ Unknown

Tubules ○ 1 ○ 2 ○ 3 ○ Unknown

Mitotic ○ 1 ○ 2 ○ 3 ○ Unknown

Lymphnodes

Number Sampled ☐☐☐

Number Positive ☐☐☐

Block1 ☐☐☐ **Block2** ☐☐☐ **Block2** ☐☐☐

7258642543

PATHOLOGY FORM-P53/BENIGN BREAST DISEASE STUDY

Henry Ford Health System

Center ID: 0 1

Study ID: ☐☐☐☐

Biopsy Date: ☐☐/☐☐/☐☐☐☐

No.of Blocks: ☐☐

No.of slides: ☐☐

Block to Send: ☐☐☐

Reserve Block: ☐☐☐

	Best Block
1. *Breast tissue present:* ☐none ☐normal	L ☐☐☐ R ☐☐☐
2. *Non-proliferative lesion:* ☐absent ☐cysts (>2mm) ☐fibrosis ☐apocrine metaplasia	L ☐☐☐ R ☐☐☐
3. *Epithelial hyperplasia without atypia:* ☐absent ☐mild ☐moderate ☐florid ☐focal (1-3 TDLU*) ☐multiple (>3 TDLU**)	L ☐☐☐ R ☐☐☐
☐columnar cell ☐focal* ☐multiple**	L ☐☐☐ R ☐☐☐
4. *Atypical Hyperplasia:* ☐absent ☐ductal ☐focal* ☐multiple**	L ☐☐☐ R ☐☐☐
☐lobular ☐focal* ☐multiple**	L ☐☐☐ R ☐☐☐
☐columnar cell ☐focal* ☐multiple**	L ☐☐☐ R ☐☐☐
☐other ☐focal* ☐multiple**	L ☐☐☐ R ☐☐☐
5. *Fibroadenoma:* ☐absent ☐simple ☐complex-no atypia ☐complex-atypia	L ☐☐☐ R ☐☐☐
6. *Sclerosing adenosis:* ☐absent ☐present	L ☐☐☐ R ☐☐☐
7. *Radial scar:* ☐absent ☐present ☐multiple (>3)	L ☐☐☐ R ☐☐☐
8. *Papillomas:* ☐absent ☐present ☐multiple (>3)	L ☐☐☐ R ☐☐☐
9. *Other:* ☐other	L ☐☐☐ R ☐☐☐

MLPA PROBE DATA FORM			
ProbeCode	PeakSize	Chromosome	HUGO
1	166	01p36.3	TNFRSF1B-D01
2	319	01p34.1	CTPS-D01
3	328	01p22-p21	F3-D01
4	472	01p13.2	NRAS-D02
5	418	01p13.2	BCAR3-D01
6	193	01q31-q32	IL10-D01
7	247	02p22-p21	MSH2-D07
8	256	02q14	IL1A-D01
9	427	02q33	ERBB4-D01
10	142	02q33	CFLAR-D01
11	220	02q24-q31	TANK-D01
12	454	03p22	CTNNB1-D01
13	337	03p21.3	MLH1-D12
14	301	03p12-p13?	IL12A-D01
15	202	03q21-q27	MME-D01
16	265	03q26.3	PIK3CA-D01
17	301	03q27	BCL6-D01
18	337	03q	Hs.222808-D01
19	418	04q22	ABCG2-D01
20	283	04q24	NFKB1-D01
21	373	04q25	CASP6-D01
22	373	04q26	IL2-D01
23	274	05q31	IL13-D01
24	154	05q31.1	IL4-D01
25	382	05q31.1-q33.1	IL12B-D01
26	142	05q	RAD17-D01
27	445	06p21.3	IER3-D02
28	184	06p21.3	KIAA0170-D01
29	238	06p21.3	BAK1-D01
30	319	06p21.3	TNF-D01
31	136	06p21.3	LTA-D01
32	328	06p21.2	CDKN1A-D01
33	427	06p21.2	CDKN1A-D02
34	418	06p21	Hs.89125-D01
35	382	06p12	VEGF-D02
36	247	06q22	MYB-D01
37	166	07q21	ABCB1-D01
38	160	07q31	MET-D01
39	427	07q35	CASP2-D01
40	400	08q11	PRKDC-D01
41	472	08q24	PTK2-D01
42	238	08q24.12	MYC-D01
43	154	08q24.12	MYC-D02
44	220	08q24.2-q24.3	SLA-D01
45	211	08q24.3	RECQL4-D01
46	346	08q24.3	PTP4A3-D03
47	373	8	FGFR1-D03
48	211	09p21	CDKN2B-D01
49	202	09p21	CDKN2A-D01

ProbeCode	PeakSize	Chromosome	HUGO
50	463	10p14-p13	RENT2-D01
51	136	10p12.1-p11.1	CREM-D01
52	148	10q23.3	PTEN-D01
53	337	11p15.5	HRAS-D01
54	193	11p13	HIPK3-D01
55	472	11p12	EHF-D01
56	409	11pter-p13	CD44-D01
57	175	11q13	RELA-D01
58	202	11q13	CCND1-D01
59	292	11q13	CCND1-D02
60	148	11q13	FGF3-D01
61	229	11q13	EMS1-D01
62	184	11q22.2-q22.3	CASP1-D01
63	310	11q22.2-q22.3	IL18-D01
64	355	11q22-q23	BIRC2-D01
65	247	11q22-q23	ATM-D01
66	436	11	LMO2-D01
67	355	12p13	CCND2-D01
68	445	12p13	TNFRSF7-D01
69	175	12p13	TNFRSF1A-D01
70	211	12q14	IFNG-D01
71	364	12q14.3-q15	MDM2-D01
72	463	12q24.13	BCL7A-D02
73	310	12	LRMP-D01
74	355	13q12.3	BRCA2-D01
75	160	13q14	RB1-D01
76	220	13q14	RB1-D02
77	148	13q32	ABCC4-D01
78	445	13q34	ING1-D01
79	238	14q12-q21.3	TINF2-D01
80	391	14q13	NFKBIA-D01
81	400	15q13	MESDC1-D01
82	194	15q21-q22.2	B2M-D01
83	454	15q25-q26	IGFIR-D01
84	346	16	MVP-D01
85	382	16q22.1	CDH1-D01
86	391	17p13.3	CRK-D01
87	346	17p13.1	TP53-D01
88	130	17p13.1	TP53-D02
89	463	17p13.1	TP53-D04
90	229	17q11-q21	SCYA3-D01
91	256	17q12-q21	TOP2A-D01
92	274	17q21	BRCA1-D13A
93	283	17q21	BRCA1-D16A
94	409	17q21.1	ERBB2-D01
95	142	17q21.1	ERBB2-D02
96	310	17q23-q24	AXIN2-D01
97	454	17q25	TIMP2-D01
98	436	18p11.32	DCC-D02
99	175	18q21	PMAIP1-D01

ProbeCode	PeakSize	Chromosome	HUGO
100	319	18q12.1	CDH2-D01
101	274	18q21.2	BCL2-D01
102	256	18q21.3	BCL2-D02
103	130	18q21.3	BCL2-D03
104	166	19p13.3-p13.2	DNMT1-D01
105	265	19p13	CDKN2D-D01
106	391	19q13	KLK3-D02
107	301	19q13.3-q13.4	BAX-D01
108	265	20p11.2	THBD-D01
109	160	20q11.1	BCL2L1-D01
110	436	20q12	NCOA3-D01
111	400	20q13	STK15-D01
112	364	20q13.1-q13.2	PTPN1-D01
113	364	21q11	STCH-D01
114	136	21q22.2	SIM2-D01
115	292	21q22.3	TFF1-D01
116	292	22q11.23	MIF-D01
117	229	Xp22.2-p22.1	PPEF1-D01
118	409	Xq25-q26	PDCD8-D01
119	283	Xq28	FMR2-D01
120	154	Xq	AR-D01
121	328	Yq11	SRY-D01
122	184	Yq11	UTY-D01

ORIGINAL ARTICLE

Delineating Genetic Pathways of Disease Progression in Head and Neck Squamous Cell Carcinoma

Maria J. Worsham, PhD; Gerard Pals, PhD; Jan P. Schouten, PhD; Rosalind M. L. van Spaendonk, PhD; Adriane Concus, MD; Thomas E. Carey, PhD; Michael S. Benninger, MD

Objective: To identify altered gene targets that characterize disease progression in squamous cell carcinoma (SCC) of the head and neck (HNSCC). Genetic alterations in HNSCC cell lines reflect the tumor in vivo and can serve as valuable tools to study the development and progression of HNSCC. Identification of key molecular events may be useful for more accurate distinction of prognostic groups for selection and targeting of therapy.

Design: Individual gene loci were analyzed for genetic alterations using a novel genomewide strategy.

Subjects: Head and neck squamous cell carcinoma primary (A) and recurrent or metastatic (B) cell lines UMSCC-11A/11B, UMSCC-17A/17B (previously karyotyped), and UMSCC-81A/81B are described.

Results: At the genome level, loss and gain of genetic loci concurred with tumor karyotypes. Several abnormal gene loci not apparent by cytogenetics were also identified. All except 11B indicated loss of *CDKN2A* (encodes p14 and p16), with concomitant loss of *CDKN2B* (encodes p15) in 11A, 17B, and 81A. All 6 cell lines showed gain of *PIK3CA* (encodes a PI3 kinase) located at 3q26.3.

Conclusions: We provide evidence for the role of 3 critical pathways in the development and progression of HNSCC. The *CDKN2A/B* genes encode various components of the Rb and p53 pathways, and the *PIK3CA* gene makes a catalytic subunit of the protein phosphatidylinositol 3-OH kinase (PI3K), which is known to be involved in the PI3K/ATK signaling pathways. Molecular events may ultimately serve to achieve genomic alterations that set off an interplay among key gene loci along discrete genetic pathways used by tumor cells in HNSCC.

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From the Research Division (Drs Worsham and Concus), Department of Otolaryngology–Head and Neck Surgery (Drs Concus and Benninger), Henry Ford Health System, Detroit, Mich; Department of Clinical Genetics, VU Medical Center, Amsterdam, the Netherlands (Drs Pals and van Spaendonk); Microbiology Research Center–Holland, Amsterdam (Dr Schouten); and Department of Otolaryngology–Head Neck Surgery, University of Michigan, Ann Arbor (Dr Carey). *The authors have no relevant financial interest in this article.*

SQUAMOUS CELL carcinoma (SCC) of the head and neck (HNSCC) is the sixth most common malignant disease worldwide. Despite advances in chemotherapy and radiation therapies, HNSCC carries a high mortality rate. The American Joint Committee on Cancer clinical tumor/node/metastasis (TNM) staging system[1] is the most important prognosticator of survival at present. Nevertheless, problems with detection of occult metastases in the neck and the fact that tumors with clinically equivalent stages may behave biologically very differently, suggest that additional prognostic markers should be used to supplement TNM staging. Because of the advances of the Human Genome Project, there is presently a unique opportunity to identify new molecular biological markers for this purpose. In addition, study of gene alterations in cancer has led recently to the development of targeted therapies in head and neck cancer, for example, to the use of monoclonal antibodies against overexpressed EGFR (C225).[2] Tumor repopulation after treatment, with the regrowth of clones resistant to apoptosis also represents an important challenge to effective long-term therapy for head and neck cancer.[3-5] This can be problematic owing to tumor heterogeneity and the fact that standard histological methods can only allow examination of limited cross sections of tumor and surrounding tissue. Identification and analysis of gene changes in cancer may additionally address each of these problems.

Genetic alterations in HNSCC cell lines reflect the tumor in vivo and can serve as valuable tools to study the development and progression of HNSCC.[6,7] The HNSCC karyotype is typically very complex,[8] but common features in SCC at one anatomic site are often very similar to SCC at other anatomic sites such as the esophagus,[9] skin,[10] and vulva,[11] irrespective of the initiating changes (eg, tobacco and alcohol, pan, or human papillomavirus). These

common changes strongly suggest that initiation, development, and progression of squamous epithelial neoplasia evoke common genetic pathways irrespective of anatomic site.

Chromosome aberrations have been landmarks to identify cancer genes in many tumor types; however, individual gene loci altered in tumors cannot be deduced solely from the type of chromosome rearrangement. We developed the multiplex ligation-dependent probe amplification (MLPA) assay (**Figure 1**), a novel genomewide strategy to identify specific gene loci for amplification and loss. We interrogated 6 HNSCC cell lines against a selected panel of 96 unique gene loci implicated in cancer and distributed throughout the genome.[12] Molecular fingerprints identified from our genomewide studies concur with chromosomal aberrations and provide a novel index to estimate the extent of genomic abnormality with disease progression.

We provide evidence for the role of 3 critical pathways in the development and progression of HNSCC. We present data that substantiates loss of *CDKN2A/B*, which encode various components of the Rb and p53 pathways[13] against a background of apparently cytogenetically normal chromosome 9p21 regions. We also show gain of *PIK3CA*, a gene located at 3q26.3 that has been shown to predict clinical outcome for early disease HNSCC tumors[14] and functions as part of a lipid-signaling pathway involved in multiple cancer-related functions.[15,16]

METHODS

HNSCC CELL LINES

Tumor sample acquisition, tissue culture, and karyotype analysis methods have been detailed elsewhere.[17,18] The UMSCC-11A and -11B cell lines were derived from tumor tissue obtained from the primary tumor site (hypopharynx) before and after chemotherapy, respectively. The SCC cell lines 17A (supraglottis) and 17B (neck soft tissue) and UMSCC-81A (left false vocal cord) and -81B (right false vocal cord) were derived from tumor tissue obtained simultaneously from primary (A) and metastatic (B) sites. The UMSCC-81A and -81B cell lines have not been cytogenetically characterized.

DNA EXTRACTION

From the 6 cell lines, DNA was extracted using the QIAamp Kit (Qiagen Inc, Chatsworth, Calif) at passages 83 and 90 for 11A and 11B, respectively; 138 and 184 for 17A and 17B, respectively; and 24 and 129 for 81A and 81B, respectively.

THE MLPA TECHNIQUE

The MLPA assay is a new method for relative quantification of approximately 40 different DNA sequences in a single reaction requiring only 20 ng of human DNA. The MLPA assay has been successfully used for the detection of deletions and duplications of complete exons in the human *BRCA1*, *MSH2*, and *MLH1* genes, detection of trisomies such as Down syndrome, characterization of chromosomal aberrations for gains and losses of genes in cell lines and tumor samples, and relative quantification of messenger RNAs.[12] Probes added to the samples are amplified and quantified instead of target nucleic acids. Amplification of

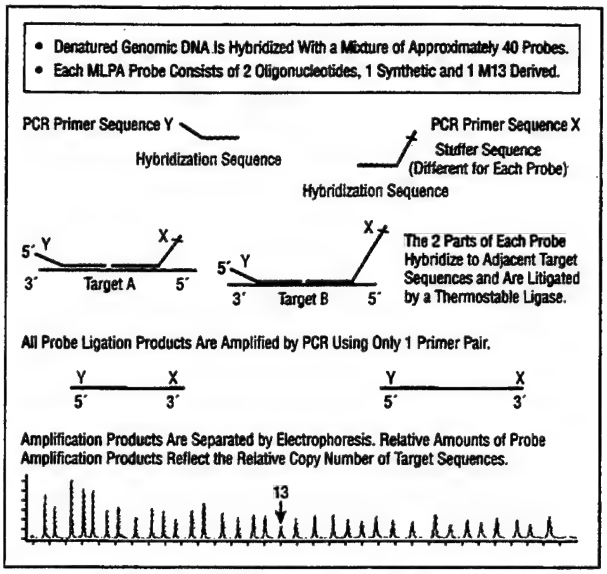

Figure 1. Multiplex ligation-dependent probe amplification (MLPA). PCR indicates polymerase chain reaction.

probes by polymerase chain reaction depends on the presence of probe target sequences in the sample. Each probe consists of 2 oligonucleotides (1 synthetic and 1 M13 derived), each hybridizing to adjacent sites of the target sequence. Such hybridized probe oligonucleotides are ligated, permitting subsequent amplification (Figure 1). All ligated probes have identical end sequences, permitting simultaneous polymerase chain reaction amplification using only 1 primer pair. Each probe gives rise to an amplification product of unique size between 130 and 480 base pairs. Probe target sequences are small (50-70 nucleotides). The prerequisite of a ligation reaction provides the opportunity to discriminate single nucleotide differences. The amplified fragments are separated on a DNA sequencer.

One of the applications of the MLPA method is the detection of chromosomal aberrations in DNA samples from tumors. This high throughput approach detects aberrant loci at 112 human genome sites.[12] These gene loci were selected because of their reported involvement in cancer and span all 23 chromosomes including the X and the Y. The quantitative nature of the results allows the detection of loss of a gene copy (loss of heterozygosity) without the need for informative heterozygous markers.

INTERPRETATION

Normal tissue from each cancer subject serves as an internal reference when available. For cell lines, where normal DNA is not available, control (normal) male and female DNA samples are run with each probe set. Quantification and loss or gain of gene loci is determined through a process of normalization. The latter addresses variations in the surface area of a peak (intensity) encountered due to fluctuations in the assay run such as amount of DNA, ploidy variations, and polymerase chain reaction conditions. Briefly, the peak area for each probe is expressed as a percentage of the total surface area of all peaks of a sample in an assay run (**Figure 2** [1C mix] and **Figure 3** [2C mix]). Relative copy number for each probe is obtained as a ratio of the normalized value for each locus (peak) of the sample to that of the normal control. A difference is significant only if the ratio is less than 0.7 (loss) or higher than 1.3 (gain). Complete loss or 0 copies is indicated by absence of a peak for that particular locus (illustrated for the loss of the *CDK2NA* at 9p21 [Figure 2] and *CDKN2B* at 9p21 and *SRY* at Yp11.2 [Figure 3]).

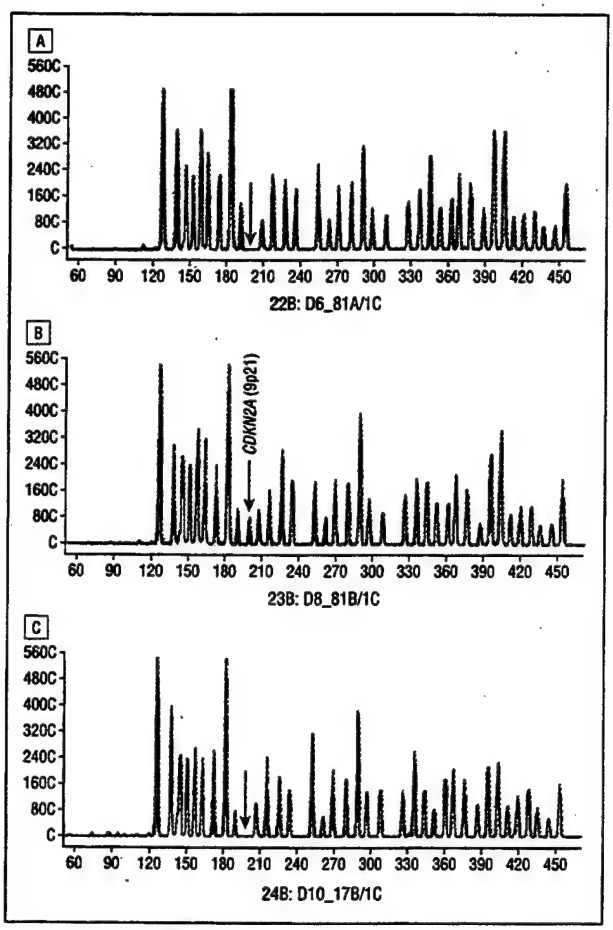

Figure 2. Multiplex ligation-dependent probe amplification assay results of probe mix 1C. Note complete loss of the *CDKN2A* gene in 81A and 17B, with retention of the gene in 81B.

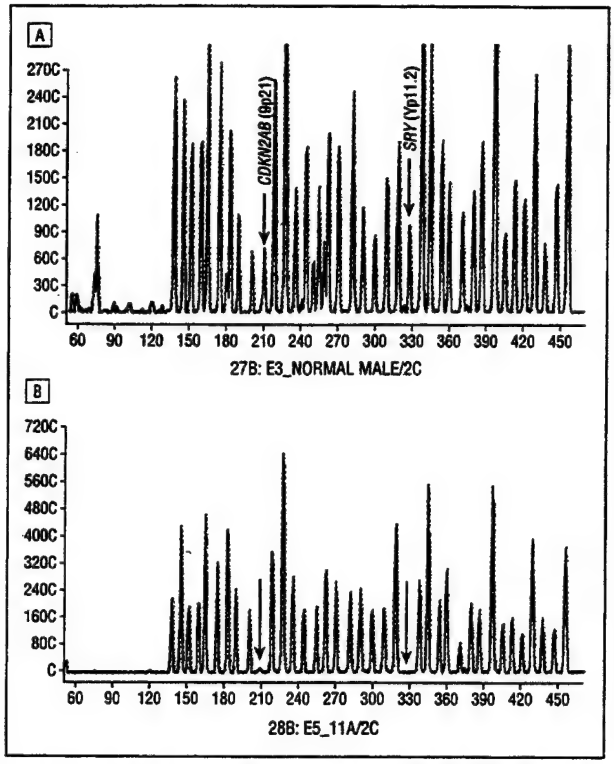

Figure 3. Multiplex ligation-dependent probe amplification assay results of probe mix 1C. Note complete loss of *CDKN2B* and *SRY* genes in 11A. The normal male control retains both gene loci.

A relative copy number of 2 is considered normal, 1 or 0 copies is considered loss, and 3 copies or more is considered gain.

The cytogenetic interpretation for gains and losses has been previously described.[8] Briefly, loss of a chromosome or segment was defined as presence of only 1 copy against a near diploid background or presence of 1 or 2 copies against a 3N (triploid) or 4N background (near tetraploid). Gain was defined as presence of at least 3 copies against a diploid background, presence of at least 5 copies against a near triploid background, and presence of at least 6 copies against a near tetraploid background.

RESULTS

HNSCC CELL LINES

Complete karyotypes for UMSCC-11A/B and -17A/B have been previously published.[17,18] The UMSCC-11A cell line had a consensus modal number of 88 chromosomes (hypotetraploid), and UMSCC-11B had a consensus modal number of 51 chromosomes (hyperdiploid). The UMSCC-17A and -17B cell lines were both hyperdiploid with consensus modal numbers of 47-49 and 47, respectively.

Overall, at the genome level, loss and gain of genetic loci concurred with tumor karyotypes (**Table**). Chromosome 3 aberrations in 11A, 17A, and 17B showed gain of 3q, which was supported by increased copy number (3-4 copies) of *PIK3CA* at 3q26.3. The *PIK3CA* gene, located at 3q26.3, indicated gain of copy number in all of the cell lines analyzed (**Figure 4**). Gain of this region of 3q is also internally validated by gain of *MME* and *BCL6* genes at 3q27 (Table). Loss of the *SRY* gene at Yp11.2 in 11A and 11B is consistent with the cytogenetic observation of lack of the Y chromosome. Loss of *SRY* was also observed in 81A; however, 81B had 2 *SRY* copies (Figure 1). The UMSCC-17A and -17B cell lines were female derived.

The *BCL2* locus interrogated in separate probe mixes indicated loss (1 copy) of this gene in 11B, concordant with 11B tumor karyotypes (Table). The UMSCC-17A and -17B cell lines had 2 copies of chromosome 18, with loss of the *BCL2* locus, which was also lost in 81A and 81B (Table).

Cytogenetics and MLPA results were discordant for *CDKN2A/B* genes at 9p21 (Figure 4). Both loci were completely lost in 11A, 17B, and 81A. This loss indicates a homozygous deletion of the 9p21 region harboring the *CDKN2A/B* genes in a background of 3 copies of chromosome 9 in 11B and 2 copies in 17B. The UMSCC-17A and -81B cell lines retained both copies of *CDKN2B*, but lost 1 copy of *CDKN2A* (Table).

COMMENT

Aberrant cancer genes rarely function independently, and evidence points to a carefully choreographed interplay among gene products in the regulation of normal growth. As an example, to progress through the cell cycle, mammalian cells must overcome the quiescent state of G_0 and

Head and Neck Squamous Cell Carcinoma Tumor Karyotypes and MLPA Relative Copy Number

		11A		11B		17A		17B		81A	81B
Probe	Chrom Position	Karyo (p38; M = 88)	MLPA (P83; 2n)	Karyo (p51; M = 51)	MLPA (P90; 2n)	Karyo (p65; M = 47-49)	MLPA (P138; 2n)	Karyo (P13, 17; M = 52, 47)	MLPA (P184; 2n)	MLPA (p24;2n)	MLPA (p128;2n)
CTNNB1	03p22	4	2	2	2	2	2	2	2	2	1
IL 12A	03p12-p13	4	3	2	3	2	4	2	3	3	4
MME	03q21-q27	5	3	2	3	4	3	4	3	3	4
PIK3CA	03q26.3	5	4	2	3	4	3	4	3	3	3
BCL6	03q27	5	4	2	3	4	4	4	2	3	3
IL6	07p21	1	3	1	2	3	3	3	3	3	3
ABCB1	07q21	0	2	1	2	3	2	2	2	2	2
ABCB4	07q21.1	0	2	1	2	3	3	3	2	2	2
SEMA3C	07q21-q31	0	2	1	2	3	3	3	1	2	2
MET	07q31	0	2	1	2	3	3	3	1	2	2
CASP2	07q35	0	2	1	2	3	3	2	1	2	2
CDKN2A	09p21	3	0	3	2	2	1	2	0	0	1
CDKN2B	09p21	3	0	3	2	2	2	2	0	0	2
TRAF2	09q34	3	2	3	3	2	2	2	3	2	4
LRMP	chrom 12	4	2	2	2	2	2	2	2	2	2
TNFRSF7	12p13	4	2	2	2	2	2	2	2	2	2
TNFRSF1A	12p13	4	2	2	2	2	2	2	2	2	2
ETV6	12p13	4	2	2	2	2	2	2	2	2	2
CCND2	12p13	4	2	2	2	2	2	2	2	2	1
IFNG	12q14	4	2	2	2	2	2	2	2	2	2
MDM2-2C	12q14.3-q15	4	2	2	2	2	2	2	2	2	2
MDM2-1C	12q14.3-q15	4	2	2	2	2	2	2	2	2	2
BRCA2	13q12.3	4	2	3	2	3	2	2	2	2	2
RB1-1C	13q14	4	2	3	2	3	3	2	2	2	2
RB1-2C	13q14	4	2	3	2	3	3	2	2	2	2
CRK	17p13.3	3	3	2	2	2	2	2	2	2	2
TP53	17p13.1	3	3	2	2	2	2	2	2	2	2
SCYA3	17q11-q21	3	2	2	2	2	1	2	1	2	2
BRCA1-1C	17q21	3	2	2	2	2	2	2	2	2	2
BRCA1-2C	17q21	3	2	2	2	2	2	2	2	2	2
BRCA1-3C	17q21	3	2	2	2	2	2	2	2	2	2
ERBB2-1C	17q21.1	3	2	2	2	2	2	2	2	2	2
ERBB2-2C	17q21.1	3	2	2	2	2	2	2	2	2	2
ERBB2-3C	17q21.1	3	2	2	2	2	2	2	2	2	2
AXIN2	17q23-q24	3	2	2	2	2	2	2	2	2	2
TIMP2	17q25	3	2	2	2	2	2	2	2	2	2
BCL2-1C	18q21.2	2	2	1	1	2	1	2	1	1	1
BCL2-2C	18q21.3	2	2	1	1	2	1	2	1	1	1
SRY	Yp11.3	0	0	0	0	*	0	*	0	0	2

Abbreviations: Chrom, chromosome; Karyo, karyotype; M, modal number of chrom; MLPA, multiplex litigation-dependent probe amplification; P, passage; 2n, diploid. The asterisk indicates female patient.

enter the active G_1 cell cycle phase. Entry into cell cycle phases is prudently orchestrated throughout. The G1/S cell cycle checkpoint is regulated mainly by the cyclin-dependent kinases (CDK4 or CDK6) bound to D-type cyclins.[19] A number of negative regulators modulate CDK activity. The *CDKN2A* and *CDKN2B* genes map to 9p21 and are in tandem, spanning a region of approximately 80 kb, with *CDKN2B* located 25 kb centromeric to *CDKN2A* (**Figure 5**). The *CDKN2A* locus at 9p21 controls both the Rb pathway that regulates G_1/S-phase transition and the p53 pathway that induces growth arrest or apoptosis in response to either DNA damage or inappropriate mitogenic stimuli by generating 2 gene products.[20] The p16 protein product functions upstream of Rb, and the p14 protein blocks MDM2 inhibition of p53 activity.[21] Inactivation of the *CDKN2A* gene, which includes mutations, homozygous deletion, and promoter methylation, have been reported at varying frequencies.[21-23] Whereas *CDKN2A/p16* gene mutations selectively inactivate the Rb pathway, deletion of the *CDKN2A* locus impairs both the Rb and p53 pathways. Deletion of the *CDKN2A* locus also frequently affects the *CDKN2B* locus, which encodes p15, an important mediator of the antiproliferative effect of transforming growth factor β.[24] Somatic alterations in the *CDKN2A* gene occur in many cancer types and germ-line mutation carriers are predisposed to a high risk of pancreatic and breast cancers.[25,26] Inactivation of p16 is the most common genetic alteration in HNSCC, making it an ideal target for gene replacement.[27] Recombinant adenovirus capable of directing a high level of p16 protein expression (Ad5-p16) demonstrated a significant antitumor effect of Ad5-p16 against human HNSCC in vivo.[27]

We found complete loss (homozygous deletion) of the *CDKN2A/B* locus in 11A, 17B, and 81A, contrary to

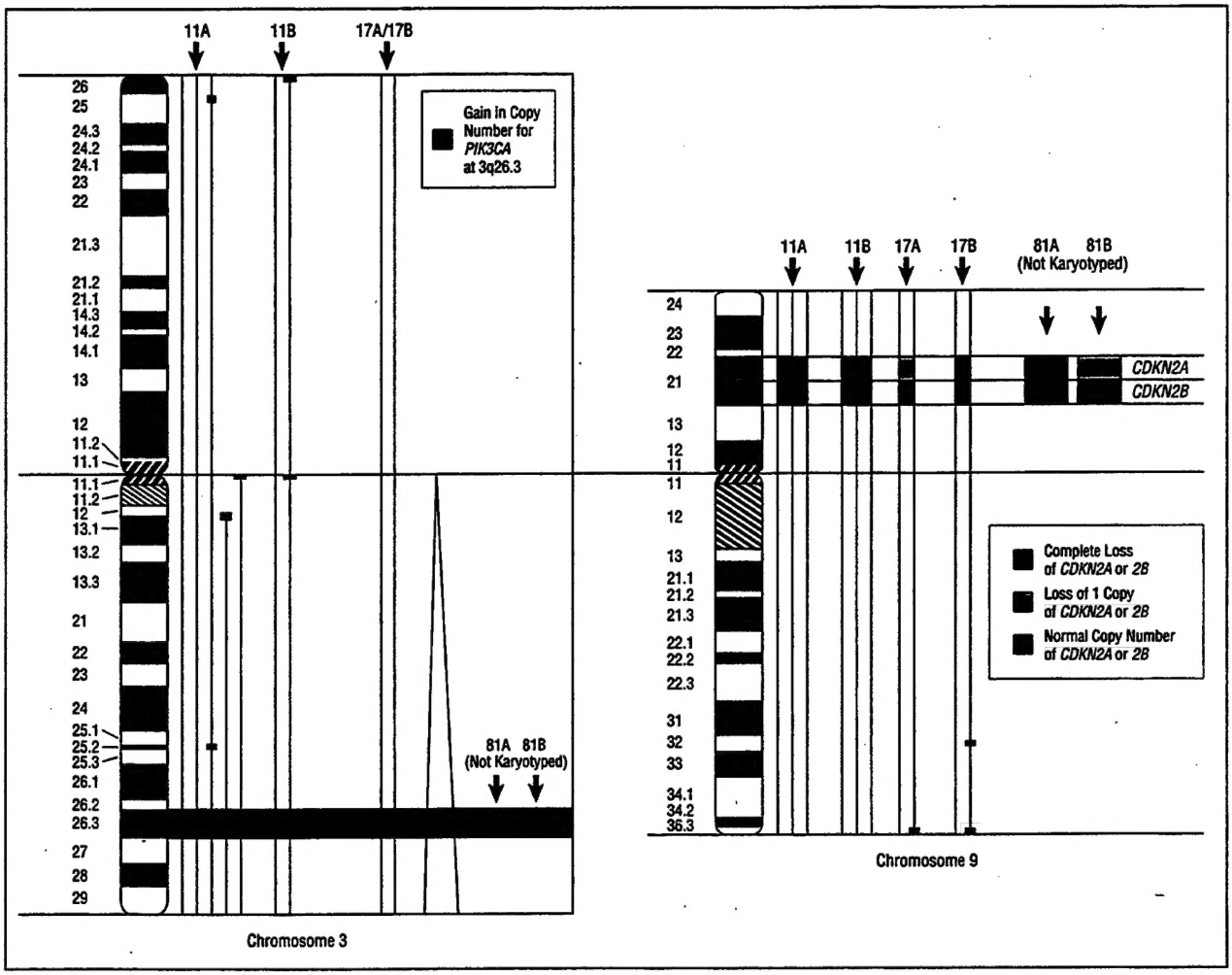

Figure 4. Ideograms representing chromosomes 3 and 9. Chromosome segments are represented as vertical solid lines. Short horizontal lines represent chromosomal break points. Chromosome 3: note 5 chromosomal copies of 3q in 11A, 2 copies in 11B, and 4 copies in 17A/B. Increased copy number of *PIK3CA* at 3q26.3 was found in all cell lines, including 81A and 81B (not karyotyped). Chromosome 9: note 3 chromosomal copies of 9, including the 9p21 locus in 11A, 3 in 11B, and 2 in 17A/B. Complete loss of *CDKN2A/B* was observed in 11A, 17B, and 81A. Note 1 copy of *CDKN2A* with retention of both copies of *CDKN2B* in 17A and 81B.

cytogenetic evidence indicating an intact 9p21 segment. The UMSCC-17A and -81B cell lines had loss of 1 copy of *CDKN2A*, but retained both copies of *CDKN2B*. Loss and retention of the *CDKN2A* locus genes *p14* and *p16* have been corroborated in another study (unpublished data, T.E.C., 2002). Cytogenetic deletion of 9p21-pter in early HNSCC has been documented[28] and suggests abrogation of this locus as an early event in HNSCC. Loss of *CDKN2A*, which results in concurrent disruption of the p16-Rb and p14-p53 pathways in several cancer types, is associated with poor prognosis, and the dual inactivation is also shown to have an obligate role in tumor suppression in animal models.[29,30]

The p53 protein, whose activity is modulated through its union to MDM2 (12q14.3-q15), also affects the G_1 to S progression by increasing expression of another CDKI, $p21^{WAF1/Cip1}$, which is able to inhibit any cyclin-CDK complexes, including D-type cyclin-CDK4/6 complexes.[20] Molecular alterations in any of these genes affect the normal G_1 to S transition and play an important role in human tumorigenesis. In this sense, mutations affecting *p53* are the most common genetic alterations yet identified in sporadic human tumors,[31] followed by genetic alterations of the 9p21 genes demonstrated in a wide variety of neoplasms.[21-23] All 6 cell lines had a normal copy number of the MDM2 gene locus (12q14.3-q15), interrogated independently in separate probe mixes (Table).

Mutation analysis of the *p53* gene[32] indicated mutant p53 in 11A and 11B (TGC→TCC, Cys→Ser in codon 242) and normal wild-type protein in 17A and 17B (p53 status not known for 81). In 11A, 3 copies of distal 17p, encompassing *p53* and *CRK* (Table), therefore, indicate gain of copy number in the presence of only mutant p53 protein. Note that cytogenetic and MLPA copy numbers were concordant for 2 copies of *p53* and *CRK* in 11B, 17A, 17B, 81A and 81B (Table). The patient designated UMSCC-11 (T2 N2a M0) with mutant p53 had a much poorer survival outcome (14 months) compared with the patient designated UMSCC-17 (T1 N0 M0), whose tumor had only wild-type p53 and who lived for over 135 months. The latter suggests that the presence of mutant p53 in addition to loss of p14 and p16 may produce even more potent growth stimuli. In an esophageal SCC cohort (OSCC), inactivation of either the *CDKN2A* or *p53*

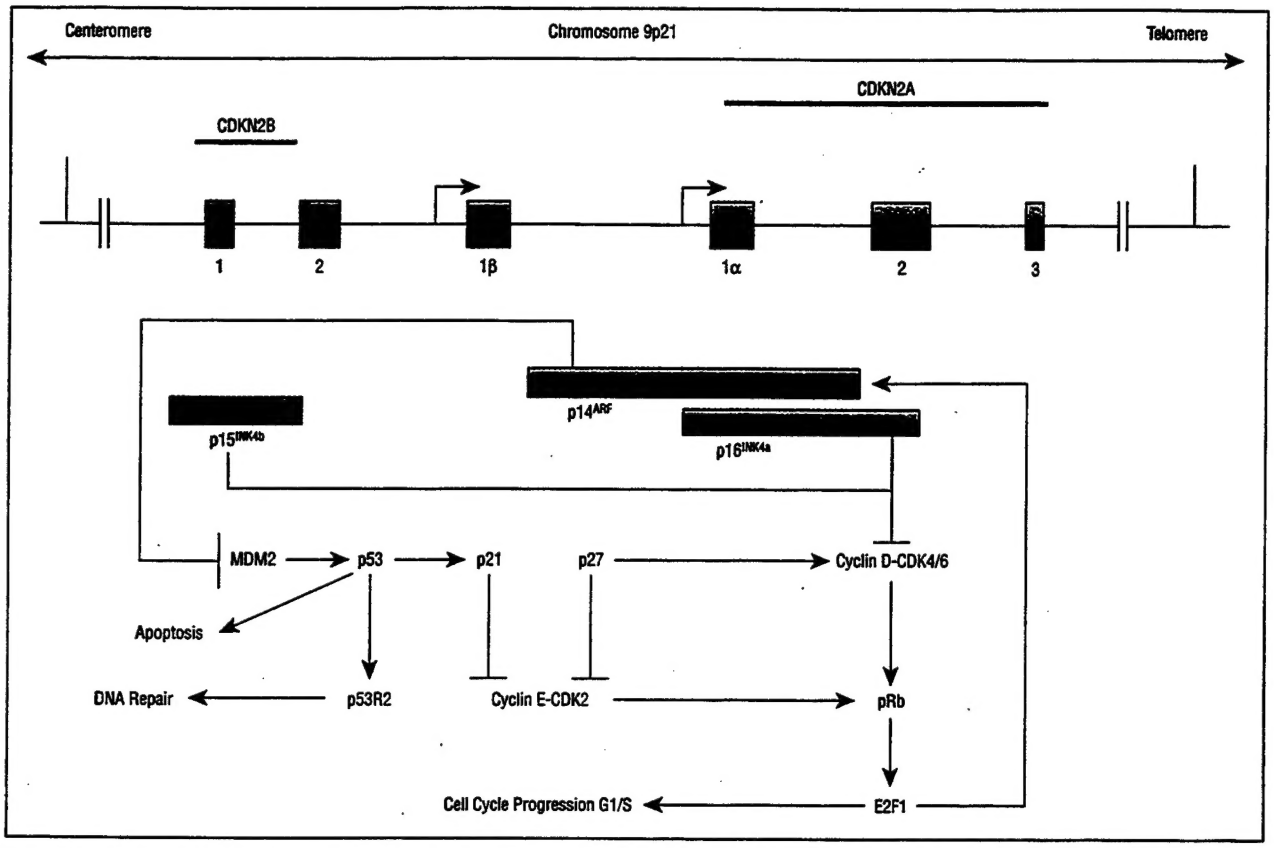

Figure 5. Genomic organization of the *CDKN2A* and *CDKN2B* genes on 9p21 (adapted from Smeds et al[33]). The p16 and p14 proteins encoded by *CDKN2A* are transcribed from separate promotors located at the 5′ side of unique exons 1α and 1β. The p15 protein is encoded by *CDKN2B*. The p16 and p15 proteins inhibit the cyclin D-CDK4/6 complex and prevent phosphorylation of Rb. The p14 protein inhibits MDM2-mediated degradation of p53. The latter functions in overlapping pathways and links Rb and p53. The stabilization of p53 mediated by p14 can lead to growth arrest via p21, apoptosis, and DNA repair.

gene in most low-grade tumors suggested the possible important and independent role of these genes in the initiation process of OSCC, whereas tumor progression probably involved acquisition of the loss of both genes.[33]

Loss of the *RB1* locus was not seen in any of the 6 cell lines. In 17B, there was gain of *RB1*, concordant with 3 intact copies of chromosome 13. In the absence of loss of heterozygosity data for the *RB1* locus, loss of 1 *RB1* allele indicative of a tumor suppressor effect cannot be ruled out.

The *PIK3CA* gene encodes a catalytic subunit of the protein phosphatidylinositol 3-OH kinase (PI3K), which is known to be involved in the PI3K/ATK signaling pathway that plays a role in multiple cancer-related functions, such as cell survival, proliferation, cell migration, vesicle trafficking, and vesicle budding.[34,35] In cervical SCC cell lines as in ovarian cancer cell lines, even low level increased *PIK3CA* copy number (ie, 3 copies) results in higher PI3K activity.[36,37] Gain of 3 to 4 copies of *PIK3CA* was noted in all 6 cell lines. Gain of this locus was validated by gain in chromosome 3 copy number in 11B, 17A, and 17B.

Recently, Redon et al[14] combined comparative genomic hybridization and fluorescent in situ hybridization coupled with quantitative polymerase chain reaction to show that *PIK3CA* (3q26.3) rather than *p63* (3q28) is a likely target of 3p26-qter amplification in low-grade HNSCC.[14] With treatment with PI3K already in place for cervical and ovarian cancer,[36,37] it would be very useful to determine how early in the tumorigenesis process amplification of *PIK3CA* occurs. Based on data from our 6 cell lines, *PIK3CA* gain was observed in all primary (A) cell lines, suggesting that gain of this gene locus is an early event.

Loss of 18q21 is one of the consistent chromosomal regions lost in HNSCC.[8] In HNSCC, loss of heterozygosity on 18q occurs frequently and has been linked to poor survival,[38,39] suggesting that 1 or more genes on this chromosome are important in tumor behavior. In our study, interrogation of the *BCL2* gene at 18q21.2 showed loss of 1 copy in all cell lines except 11A, indicating that loss of *BCL2* at 18q21.2 may be affected by 18q loss of heterozygosity. *BCL2* encodes a mitochondrial membrane protein that blocks apoptosis.

Loss of the Y chromosome in HNSCC is not an uncommon event. In a study of 29 cases of HNSCC, the Y chromosome was lost from 10 of 19 tumors from male patients, and an Y rearrangement was observed in 4 others.[8] Among female patients, loss of the short arm of the inactivated X was frequent in both HNSCC and in SCC of the female genital tract. The short arm of the X and the Y chromosome shares many genes, and loss of 1 or more of these might be advantageous to the tumor cells, but both also contain late replicating regions, the loss of which have implications for faster cell cycle rates.[8] In prostate cancer, significant loss of Y chromosome–specific genes was reported, suggesting their role in pathogenesis of this disease. The loss of *SRY* and *BPY2* genes was more frequent in higher stages and grades of prostate cancer.[40,41]

Our genomewide search also indicated other changes not apparent by cytogenetics. Lack of concordance between the MLPA assay and cytogenetics, while uncommon, may be attributed in part to the ability of the MLPA assay to account for cytogenetically uncharacterized rearrangements such as marker chromosomes. For example 11A and 11B had loss of chromosome 7 accompanied by several markers chromosomes (Table) indicating that the assay can decipher and discriminate on the basis of DNA composition cytogenetically cryptic and unstable chromosomal rearrangements, especially submicroscopic deletions.

Historically, the molecular pathogenesis of cancer has been teased out 1 gene at a time. In the present study, adopting a comprehensive approach, we validated cytogenetic gains and losses uncovering specific gene loci as consistent molecular events in HNSCC.

We provide evidence for the role of 3 critical pathways in the development and progression of HNSCC. Losses of the *CDKN2A/B* genes at 9p21 encode various components of the Rb and p53 pathways. Gain of copy number of *PIK3CA* in all of the cell lines evaluated points to the involvement of the PI3K protein in lipid signaling pathways as key molecular events in both primary and metastatic disease progression. This information may be useful in molecular diagnosis, selection, and targeting of therapy in HNSCC.

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Corresponding author and reprints: Maria J. Worsham, PhD, Department of Otolaryngology–Head and Neck Surgery, Henry Ford Health System, 1 Ford Pl, Section 1D, Detroit, MI 48202 (e-mail: mworsha1@hfhs.org).

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